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Cryptic Species in the Fern *Ceratopteris thalictroides* (L.) Brongn. (Parkeriaceae). II. Cytological Characteristics of Three Cryptic Species

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Ceratopteris thalictroides has been reported to contain three cryptic species, called the south type, the north type and the third type. To obtain a clear understanding of these cryptic species, the somatic and meiotic chromosomes were examined in 18 sporophytes from different localities and three hybrids synthesized among the three types. The examinations revealed that the north type and the third type are tetraploids with $2n = 156$ and $n = 78$ chromosomes, whereas the south type is a hypotetraploid with $2n = 154$ and $n = 77$ chromosomes. Several peculiar configurations were occasionally observed at diakinesis in all three types: single rope-shaped bivalents with acrosyndesis, split bivalents with acrosyndetic connections, and quadrivalent or quadrivalent-like configurations. Occasional quadrivalents indicate the presence of several homologies within the genomes of the three types. Many univalents were observed at metaphase I in the hybrid between the south and north types, whereas only a few univalents were occasionally observed in the hybrids between the third and north types, for which a partial cross-sterility had been reported. This suggests that the chromosomal structure has differentiated little between the genomes of the third type and the north type and that the partial cross-sterility is mostly due to genic differentiation between them.

Key words: *Ceratopteris thalictroides*, cryptic species, cytology, fern, hybrid sterility, Parkeriaceae, polyploid, quadrivalent

The latest taxonomic revision of the fern genus *Ceratopteris* was carried out by Lloyd (1974). He recognized four species in the genus: *C. cornuta* (Pal. Beauv.) Le Prieur, *C. pteridoides* (Hook.) Hieron., *C. richardii* Brongn. and *C. thalictroides* (L.) Brongn. Among them *C. thalictroides* is characteristically highly polymorphic. Most of the cytological studies of *Ceratopteris* have shown that the former three species are diploids with $2n = 78$ chromosomes and the remaining one, *C. thalictroides*, is tetraploid with $2n = 156$ chromosomes (Löve *et al.* 1977). The cytological evaluation of *C. thalictroides*, however, is inconclusive, as Pal (1959) and Pal & Pal (1963) documented $2n = 80$ and Ninan (1956) and Hickok (1979) reported $2n = 154$ for this species, indicating the presence of intraspecific polyploids and aneuploids. In the previous study (Masuyama *et al.* 2002), we revealed the presence of three distinct entities in *C. thalictroides*, which we called the south type, the north type and the third type. They were regarded as independent species for two reasons. One was that they were sufficiently different in molecular characteristics, including allozyme composition and nucleotide sequences of

the chloroplast DNA. The second was that crossing tests indicated rigid cross-sterility between the south type and the other two and partial cross-sterility between the north type and the third type.

These studies lead to the following previsions with respect to the cytological characteristics of the three types: 1) they may differ not only in molecular characteristics but also in chromosome number; 2) different levels of cross-sterility among the three types may be related to different ploidy levels or to aneuploidy. The purpose of this study is to show evidence for or against these previsions by examining the chromosomes of the three types of *Ceratopteris thalictroides*.

Materials and Methods

Of the sporophytes that were used in the previous study (Masuyama *et al.* 2002), all but IN3 (Indonesia) and TW1 (Taiwan) were examined cytologically (Table 1). The Japanese sporophytes O, M, F and Y, of which the former two and the latter two were from populations with allozyme compositions of the south type and the north type, respectively (Watano & Masuyama 1994), were also examined. Additional observations were made of meiosis in the hybrids between the third and north types (IN1 × N and IN2 × N) and between the south and north types (IN4 × N) that had been synthesized in the previous study. All the sporophytes but O, M, F and Y have been cultivated successively through asexual reproduction by gemmae since the previous study. The sporophytes O, M, F and Y were obtained through mixed matings of gametophytes generating from spores, following the method described previously (Masuyama 1996). For examination of somatic

TABLE 1. Code names, localities and molecular characteristics of the sporophytes examined.

Code name[1)]	Locality	Spore collection number	Sequence of chloroplast DNA[2)]	Allozyme composition[3)]
S	Onnason, Okinawa Pref., Japan	S. Masuyama 2611	South type	South type
O	Ootomi, Iriomote Isl., Okinawa Pref., Japan	S. Masuyama 2877	–	South type
M	Motonagura, Ishigaki Isl., Okinawa Pref., Japan	S. Masuyama 2876	–	South type
TH	Khao Taln, Chumphon, Thailand	S. Masuyama 3107	South type	South type
ID	Mirzapur, Uttar Pradesh, India	S. Masuyama 2741	South type	South type
IN4	Gnung Batu, Bogor, Indonesia	S. Masuyama 2599	South type	South type
NG	Hisue, Kairuku, New Guinea	S. Masuyama 2737	South type	South type
GY	Georgetown, Guyana	S. Masuyama 2740	South type	South type
N	Nagareyama, Chiba Pref., Japan	S. Masuyama 2869	North type	North type
F	Fukiage, Kagoshima Pref., Japan	S. Masuyama 1293	–	North type
Y	Yanyu, Amami Isl., Kagoshima Pref., Japan	S. Masuyama 2131	–	North type
TW2	Tali, Taichung Pref., Taiwan	S. Masuyama 2700	North type	North type
TW3	Taichung, Taichung Pref., Taiwan	S. Masuyama 2681	–	North type
HW1	Hanapepe Riv., Kauai, Hawaii, USA	S. Masuyama 2736	–	North type
HW2	Hanalei, Kauai, Hawaii, USA	S. Masuyama 2868	North type	North type
GA	Mt. Santa Rosa, Yigo, Guam	S. Masuyama 2964	North type	North type
IN1	Gnung Sali, S. Salak, Indonesia	S. Masuyama 2701	Third type	Third type
IN2	Pasir Kuda, Bogor, Indonesia	S. Masuyama 2601	Third type	Third type

1) The same as in the previous study (Masuyama *et al.* 2002) except O,M,F and Y, new codes for additional collections.

2) See the previous study (Masuyama *et al.* 2002).

3) See the previous studies (Watano and Masuyama 1994, Masuyama *et al.* 2002).

chromosomes, roots of the sporophytes were fixed in Farmer's solution (3 anhydrous ethyl alcohol : 1 glacial acetic acid) after pretreatment with 4 mM 8-hydroxyquinoline solution for about 6 hours. For microscopic observations, the roots were put in 45% acetic acid for more than 15 minutes, followed by maceration in 1N HCl solution at 60 °C for 5 minutes. After a short rinse with 45% acetic acid, the root tip was cut off and placed in a drop of aceto-orcein (45% acetic acid containing 2% orcein) on slides. The material was spread by tapping on a cover glass with a stick and then squashed to provide stained somatic cells. For examination of meiotic chromosomes, young fertile leaves were fixed in Farmer's solution. For observation, the leaves were placed in 45% acetic acid for more than 15 minutes. Sporangia were then removed and placed in a drop of aceto-carmin (45% acetic acid containing 1% carmin) on slides and squashed under a cover glass to provide stained spore mother cells. Voucher specimens of the sporophytes examined were deposited in KYO.

TABLE 2. Chromosome numbers of sporophytes of *Ceratopteris thalictroides*.

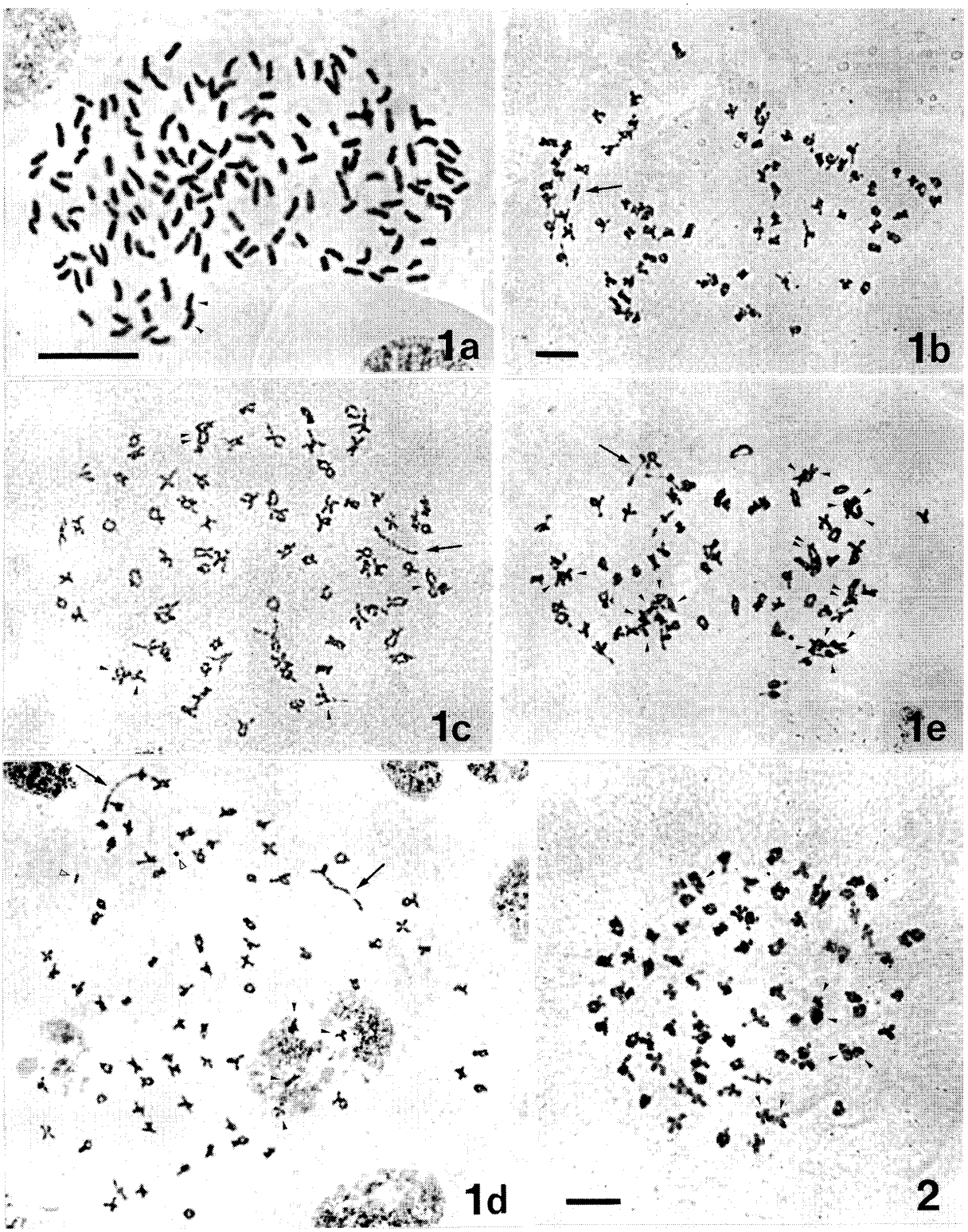
Code name	Locality	Somatic no.	Meiotic no.	Fig. in text
South type				
S	Japan	2n=154	n=77	1-a,b,c,d,e
O	Japan	2n=154	n=77	2
M	Japan	–	n=77	
TH	Thailand	2n=154	n=77	3
ID	India	2n=154	n=77	4
IN4	Indonesia	2n=154	n=77	5
NG	New Guinea	2n=154	n=77	6
GY	Guyana	2n=154	n=77	7-a,b
North type				
N	Japan	2n=156	n=78	8-a,b
F	Japan	2n=156	n=78	9
Y	Japan	–	n=78	
TW2	Taiwan	–	n=78	
TW3	Taiwan	2n=156	n=78	10
HW1	USA	–	n=78	
HW2	USA	2n=156	n=78	11
GA	Guam	2n=156	n=78	12
Third type				
IN1	Indonesia	2n=156	n=78	13-a,b
IN2	Indonesia	2n=156	n=78	14

Results

Chromosome numbers determined in this study are summarized in Table 2. Representative chromosome configurations are shown in Figs. 1 to 17. Cytological features worthy of note in the three types and the hybrids are described below.

The south type

All the sporophytes of the south type had $2n = 154$ and/or $n = 77$ chromosomes. In diakinesis of meiosis, some bivalents showed a knotted rope-like configuration with pairing at the terminal regions of arms (Fig. 1b). Occasionally, several bivalents showed a single rope-like configuration with acrosyndesis, the pairing at the ends of arms (Figs. 1c, 4 and 7b). Some of them paired further at their ends with other normal bivalents, resulting in the formation of tailed quadrivalents (Fig. 1d). There was a split bivalent with an acrosyndetic connection by a faint bridge, which appeared like two univalents (Fig. 1e). Quadrivalent-like configurations, some of which were probably true quadrivalents, were observed in many spore mother cells (Figs. 1c, 1e, 4 and 5). The occurrence of these variant configurations was dependent on the spore mother cells, not on the individual plants. Among the samples of the south type, the sporophyte of Guyana was peculiar in having a single dot-like chromosome in the somatic cells (Fig. 7a). Although the dot-like chromosome was constantly observed among 154 regular chromosomes, meiosis did not appear abnormal and 77 normal bivalents were formed (Fig. 7b).



FIGS. 1-17. Somatic and meiotic chromosomes of sporophytes of *Ceratopteris thalictroides*. Single arrowheads indicate single chromosomes in somatic division or single bivalents in meiosis; double arrowheads indicate quadrivalents or quadrivalent-like configurations. Open arrowheads show spots or foreign chromosomes. Bars indicate 10 μm; figures without bar are at the same magnification as in Fig. 1b. Fig. 1a. Metaphase of sporophyte S showing $2n = 154$ chromosomes. FIG. 1b. Diakinesis of sporophyte S showing $n = 77$ chromosomes including a knotted rope-shaped bivalent (arrow). FIG. 1c. Single rope-shaped bivalent (arrow) in diakinesis of sporophyte S. FIG. 1d. Tailed quadrivalents (arrows) in diakinesis of sporophyte S. Two bivalents are out of picture. FIG. 1e. Bivalent with acrosyndetic connection (arrow) in metaphase I of sporophyte S. FIG. 2. Metaphase I of sporophyte O showing $n = 77$ chromosomes.

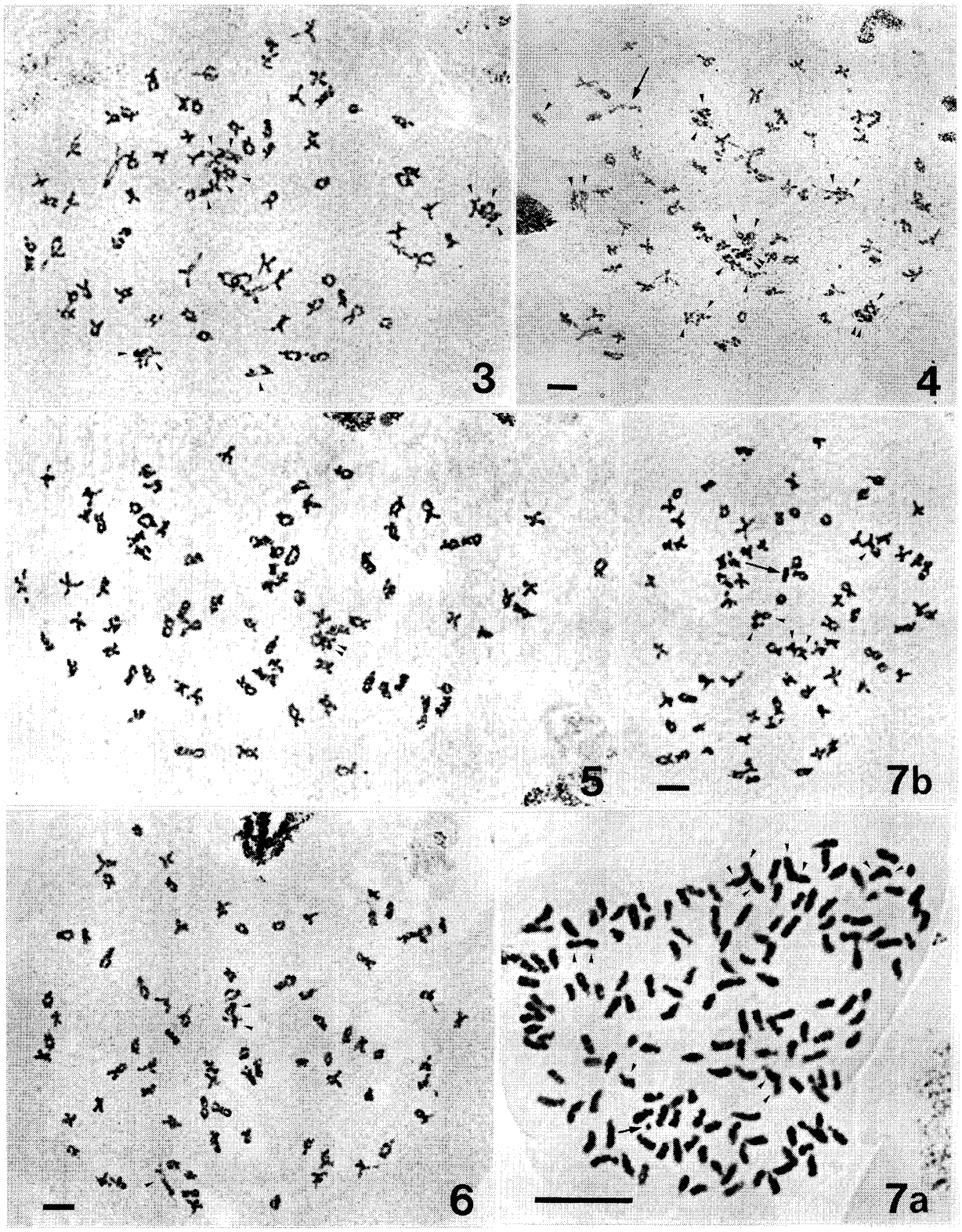


FIG. 3. Diakinesis of sporophyte TH showing $n = 77$ chromosomes. FIG. 4. Diakinesis of sporophyte ID showing $n = 77$ chromosomes including a single rope-shaped bivalent (arrow). FIG. 5. Diakinesis of sporophyte IN4 showing $n = 77$ chromosomes. FIG. 6. Diakinesis of sporophyte NG showing $n = 77$ chromosomes. FIG. 7a. Metaphase of sporophyte GY showing $2n = 154$ chromosomes and a dot-like chromosome (arrow). FIG. 7b. Diakinesis of sporophyte GY showing $n = 77$ chromosomes including a single rope-shaped bivalent (arrow).

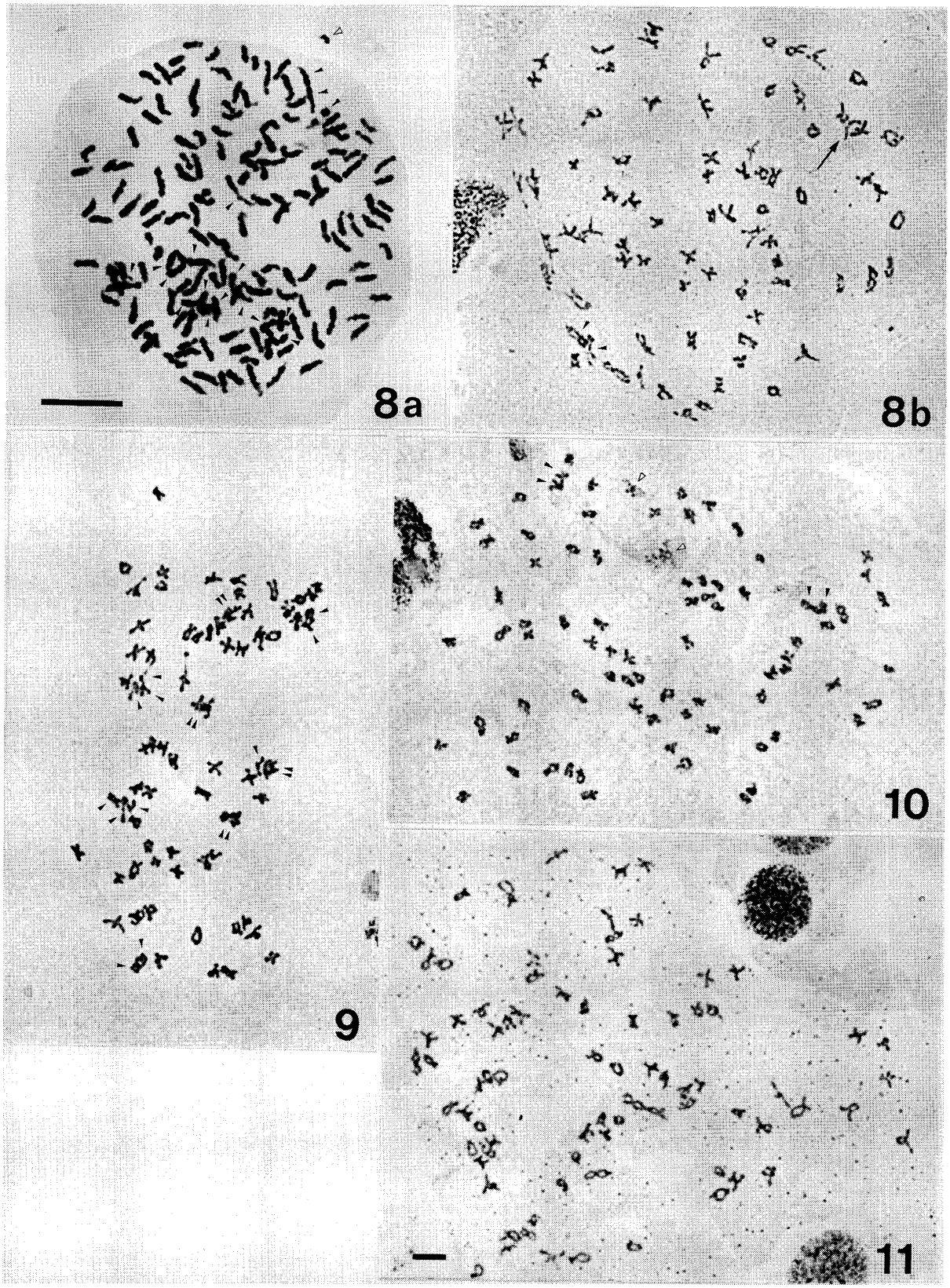


FIG. 8a. Metaphase of sporophyte N showing $2n = 156$ chromosomes. FIG. 8b. Diakinesis of sporophyte N showing $n = 78$ chromosomes including a single rope-shaped bivalent (arrow). FIG. 9. Diakinesis of sporophyte F showing $n = 78$ chromosomes. FIG. 10. Diakinesis of sporophyte TW3 showing $n = 78$ chromosomes. FIG. 11. Diakinesis of sporophyte HW2 showing $n = 78$ chromosomes.

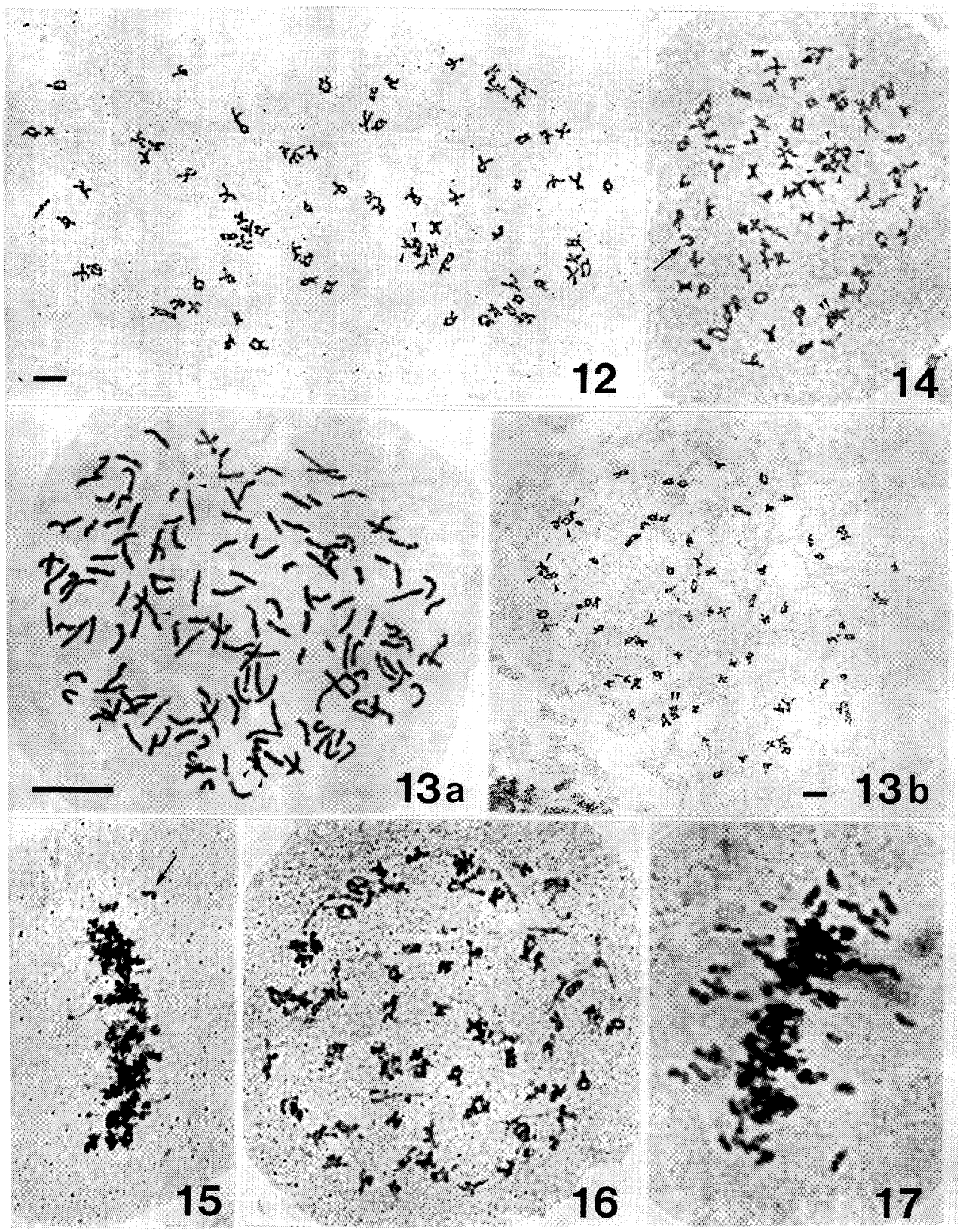


FIG. 12. Diakinesis of sporophyte GA showing $n = 78$ chromosomes. FIG. 13a. Metaphase of sporophyte IN1 showing $2n = 156$ chromosomes. FIG. 13b. Diakinesis of sporophyte IN1 showing $n = 78$ chromosomes. FIG. 14. Diakinesis of sporophyte IN2 showing $n = 78$ chromosomes including a single rope-shaped bivalent (arrow). FIG. 15. Metaphase I of the hybrid IN1 × N showing a univalent (arrow). FIG. 16. Late prophase I of the hybrid IN1 × N. FIG. 17. Metaphase I of the hybrid IN4 × N showing many univalents.

The north type

All the sporophytes of the north type had $2n = 156$ and/or $n = 78$ chromosomes. As in the south type, occasional bivalents showing acrosyndesis were observed at diakinesis in some spore mother cells (Fig. 8b). Quadrivalent-like configurations were also observed in many spore mother cells (Fig. 9).

The third type

Both sporophytes examined had $2n = 156$ and $n = 78$ chromosomes. As in the above two types, bivalents showing acrosyndesis (Fig. 14) and quadrivalent-like configurations (Figs. 13b and 14) were occasionally observed at diakinesis in meiosis.

The hybrids

Three hybrids of two combinations were examined for the occurrence of univalents at metaphase I. In the hybrid IN1×N (the third type × the north type), 40 spore mother cells (SMCs) were examined. One and two univalents were observed in 12 and 2 SMCs, respectively (Fig. 15); the remaining 26 SMCs showed no univalents. In the hybrid IN2×N (the third type × the north type), 48 SMCs were examined. One and two univalents were observed in 13 and 1 SMCs, respectively; the remaining 34 SMCs had no univalents. In both hybrids, abnormal behavior was not detected in chromosomal pairing at diakinesis (Fig. 16). In the hybrid IN4×N (the south type × the north type), a number of univalents occurred in metaphase I (Fig. 17). A rough estimation of the number of univalents was possible in 5 SMCs, where at least 22, 27, 31, 32 and 35 univalents were recognized. Pairing behavior at diakinesis was irregular, resulting in a number of univalents and multivalents among bivalents.

Discussion

Molecular analyses of the widely distributed species *Ceratopteris thalictroides* (Masuyama *et al.* 2002) revealed the presence of three distinct entities, which were called the south type, the north type and the third type. Cytological analyses in the present study showed that the sporophytes regarded as the south type had a complement of $2n = 154$ and/or $n = 77$ chromosomes without exception, while those regarded as the north type and the third type had $2n = 156$ and/or $n = 78$ chromosomes. It is evident from these results that both the north type and the third type are characterized by the chromosome number $2n = 156$ and $n = 78$, whereas the south type is characterized by $2n = 154$ and $n = 77$. In an experimental study utilizing several sporophytes of the south type and the north type, Masuyama & Watano (1994) reported both types to have the same chromosome number, $2n = 156$. It appears that insufficient observations resulted in a miscount for the south type. Since the chromosome number of $2n = 78$ has been reported for diploid species of *Ceratopteris* (Löve *et al.* 1977), the count of $2n = 156$ in the north type and the third type is tetraploid and the count of $2n = 154$ in the south type is a hypotetraploid.

Ninan (1956) reported chromosome counts of $2n = 154$ and $n = 77$ for *Ceratopteris thalictroides* in India. Later, in a cytological study of seven different collections of *C. thalictroides*, Hickok (1979) reported $n = 77$ for six collections and $n = 78$ for one collection. The six collections with $n = 77$ contained those from Malaysia and Surinam, localities not included in this study. The collection with n = 78 was from Hawaii, the locality included in this study. Combining these cytological data with the results of this study, it appears that the south type with 2n = 154 and n = 77 is distributed widely in tropical and subtropical regions, and the north type with $2n = 156$ and n = 78 ranges from eastern Asia to Micronesia, although their distribution in Africa is uncertain. The third type, another strain of $2n = 156$, appears to be narrowly distributed, occurring only in Indonesia and neighboring areas.

Pal (1959) reported the chromosome number $2n = 80$ and $n = 40$ for an Indian sporophyte iden-

tified as *Ceratopteris siliquosa*, a synonym of *C. thalictroides*. Later, Pal & Pal (1963) also reported the same chromosome number for *C. thalictroides* in India. Judging from the number reported, the sporophytes examined appear to represent a strain derived from a diploid species of *Ceratopteris*.

In meiosis in the three types, two peculiar configurations of bivalents were occasionally observed: single rope-shaped bivalents with acrosyndesis (Figs. 1c, 4, 7b, 8b and 14) and split bivalents with an acrosyndetic connection (Fig. 1e). The single rope-shaped bivalents appeared to be transformed configurations of knotted rope-shaped bivalents (Fig. 1b) after terminalization of chiasma. Similarly, the split bivalents appear to result from occasional transformation of single rope-shaped bivalents.

In addition to these peculiar bivalents, quadrivalent-like configurations (Fig. 1c, for example), including tailed ones (Fig. 1d), were occasionally observed. Occasional quadrivalents have been reported for synthesized hybrids between diploid species of *Ceratopteris* (Hickok & Klekowski 1974, Hickok 1977) and crosses among different collections of *C. thalictroides* (Hickok 1979). Most of the sporophytes utilized in the present study, however, were not crosses but ordinary individuals, because it was reported that their selfed progeny formed viable spores at sufficiently high frequencies (Masuyama *et al.* 2002). Occasional quadrivalents, therefore, appear not to be exclusive to crosses between different species and forms, but to be rather common in the tetraploid species of *Ceratopteris*. Occasional quadrivalent formation in the three types indicates that some of different pairs of homologous chromosomes are of homoeologous origin and still maintain homologies at several sites. In seed plants, occasional quadrivalent formation has been frequently observed in polyploid taxa and explained in terms of the mechanism leading to stable bivalent formation in polyploids (Driscoll *et al.* 1980, Watanabe 1981, Jackson & Casey 1982).

The sporophyte from Guyana is characterized by the consistent occurrence of a single dot-like chromosome among somatic chromosomes (Fig. 7a). The dot-like chromosome appears to be a B-chromosome because 77 normal bivalents were constantly observed in meiosis (Fig. 7b). It is uncertain whether the dot-like chromosome is characteristic of plants in Guyana or is only in the individual examined.

Mating tests in our previous study (Masuyama *et al.* 2002) demonstrated that when S (a sample of the south type, see Table 1) was utilized as a paternal tester, spores of hybrids synthesized with the north type and those with the third type showed 0 to 18% and 2 to 20% germination, respectively, but when N (a sample of the north type) was paternal, the spores of hybrids with the south type and those with the third type showed 0 to 20% and 37 to 69% germination, respectively. As selfed progeny of each type showed 83 to 98% spore germination, it was concluded that the spore germination rates of hybrids noted above represented rigid cross-sterility between the south type and the other two types and partial cross-sterility between the third type and the north type (Masuyama *et al.* 2002). In the present study, a large number of univalents consistently occurred in metaphase I in the hybrid between the south type and the north type (Fig. 17). This implies that, in addition to chromosome number, chromosomal structure and genic constitution have considerably differentiated between the genomes of these types and have resulted in their rigid cross-sterility. In contrast, one or two univalents occasionally occurred in the hybrids between the third type and the north type (Fig. 15). In a cytological study of synthesized hybrids among diploid species of *Ceratopteris*, Hickok (1977) reported that the hybrids showed 23 to 30% spore germination and a few univalents occurred only in less than 8% of the spore mother cells examined, a feature similar to that in the above hybrids between the third type and the north type. From this pairing behavior he concluded that spore abortions of the hybrids were

probably caused not by chromosomal but by genic imbalance in the spores after meiotic segregation. The same conclusion can be drawn for the above hybrids in this study. That is, chromosomal structure may have differentiated little between the genomes of the third type and the north type and the partial sterility of the hybrids may be due mostly to different genic constitutions of these types.

As noted in the beginning, the south type of $2n = 154$ is regarded as hypotetraploid with a decreased number of chromosomes. Two processes can be supposed in the formation of this aneuploid. One is the numerical loss of chromosomes by fusion of two homologous pairs after formation of tetraploids with $2n = 156$ chromosomes. Particular configurations indicative of chromosomal fusion, however, were not evident in somatic and meiotic chromosomes of the south type. The other process is allotetraploid formation through hybridization between ordinary diploids with $2n = 78$ and unknown diploids with $2n = 76$. Pal (1959) and Pal & Pal (1963) reported a chromosome number $2n = 80$ for Indian sporophytes of *Ceratopteris*. Their findings suggest that several cytological variants may be present among diploid species of *Ceratopteris*, although a chromosome number $2n = 78$ has been exclusively recognized for diploid species so far (Löve *et al.* 1977). Further cytological study of diploids in *Ceratopteris* may reveal an outline of the origin of the south type and the other two types in *C. thalictroides*.

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